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# The C-terminal domain of peptide deformylase is disordered and dispensable for activity

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**Abstract** **Upon trypsinolysis, the 18 C-terminal residues of *Escherichia coli* peptide deformylase were removed but the resulting form exhibited full activity. Moreover, a mutant *fms* gene encoding the first 145 out of the 168 residues of the enzyme was able to complement a *fms*(Ts) strain and exhibited full activity. Upon progressive truncation up to residue 139, both activity and stability decreased up to complete inactivation. Mutagenesis of residues of the 138–145 region highlights the importance of Leu-141 and Phe-142. N-Terminal deletions were also carried out. Beyond two residues off, the enzyme showed a dramatic instability. Finally, NMR and thermostability studies of the full-length enzyme and comparison to the 1–147 form strongly suggest that the dispensable residues are disordered in solution.**

*Key words:* Domain architecture; Deletion; Complementation; NMR; Active site

## 1. Introduction

In bacteria, because methionyl-tRNA$^{fMet}$ needs the addition of an *N*-formyl group to initiate translation [1,2], all nascent polypeptide chains start with *N*-formylmethionine. Still, mature proteins generally do not retain the *N*-formyl moiety (see [3] for a review). The removal of this formyl group is ensured by peptide deformylase (PDF, EC 3.1.1.29), an enzyme releasing formate and deformylated peptides as products [4]. The *fms* genes encoding *Escherichia coli* and *Thermus thermophilus* PDF are cotranscribed with those of methionyl-tRNA$^{fMet}$ formyltransferase and encode enzymes of 168 and 191 amino acids, respectively [1,5-7]. *E. coli* PDF is a monomer with one strongly bound zinc ion [7,8]. In addition, the HEXXH motif characteristic of zinc peptidases was noticed in the amino acid sequences of the *E. coli* and *T. thermophilus* enzymes. Systematic substitutions of the residues of the *E. coli* enzyme possibly involved in zinc binding established that the zinc ion is coordinated by both histidines of the $_{132}$HEXXH$_{136}$ motif and that $E_{133}$ was essential for deformylation activity [9]. PDF thus resembles other known zinc metalloproteases such as astacin, thermolysin or collagenases. Surprisingly, $C_{90}$, the third chelator of the zinc atom of PDF, is located at the N-terminal side of the HEXXH motif [9]. PDF therefore markedly differs from the already described members of the zinc metalloproteases superfamily whose third zinc chelator is a histidine or glutamate residue systematically located at the C-terminal side of the HEXXH motif (see [10,11] for reviews).

Since these features support the idea that PDF could be part of a new type of zinc metalloproteases [9], it was of interest to further characterize the structure-function relationships of this protein. A deletion analysis was therefore carried out with *E. coli* PDF as starting enzyme. The obtained results show that, contrarily to the N-terminal residues which are not dispensable, the last 23 may be omitted without loss of activity. Strong arguments favouring the idea that these residues are disordered in the 3D structure are given.

## 2. Materials and methods

### 2.1. General techniques

Mutations were introduced by oligonucleotide site-directed mutagenesis [12] of M13def and the pUCdef variants were reconstructed as previously reported [9]. DNA sequences of the mutated genes were determined by the dideoxy chain-termination method [13] by using the automatic ALF system (Pharmacia).

Peptide deformylase activity was measured as previously described [9,14]. Zinc content was determined by atomic absorption spectroscopy using a Varian AA775 spectrophotometer equipped with an air-acetylene burner [15]. Western blot analysis was performed by using specific antibodies against PDF as described [9].

### 2.2. Purification of PDF variants

PAL421Tr cells [6] carrying the mutant pdef-Ec were used to inoculate a flask of 2×TY medium (0.5 liter) containing 50 μg/ml ampicillin and 0.5 mM IPTG. Growth was carried out for 15 h at 37°C. Cells were harvested by centrifugation and resuspended in 40 ml of 20 mM $KH_2PO_4$ pH 7.0 (buffer A). The sample was sonicated, and cell debris removed by centrifugation. The preparation of the crude extract as well as the chromatographic steps were as previously described [7] except that a concentration of 3% (w/v) streptomycin sulfate was used instead of 0.3%. In the case of the truncated variants of PDF, the hydrophobic interaction column was replaced by a molecular sieving step on a Superdex-75 column (60×1.6 cm; Pharmacia). Protein samples (10 ml) were loaded on the column and further eluted with 50 mM $KH_2PO_4$ (pH 7.0) at a flow rate of 0.5 ml/min. The proteins were recovered homogeneous and stored at −30°C in buffer A plus 55% glycerol. A calculated molar extinction coefficients of 2360 $M^{-1}\cdot cm^{-1}$ was used to determine the concentrations of the studied *E. coli* PDF variants.

### 2.3. Mild trypsinolysis

Trypsinolysis of PDF variants was performed at 37°C in 0.1 M Tris-HCl (pH 7.7), 10 mM EDTA and 1 mM 2-mercaptoethanol. The proteolysis was initiated by the addition of increasing amounts of TPCK-treated trypsin from bovine pancreas (Sigma). Samples were usually incubated for 15 min, and the trypsinolysis was quenched by the addition of chicken eggwhite ovomucoid (Sigma; 2/1 (w/w) with respect to trypsin). The reaction products were analyzed by SDS/PAGE on 20% homogeneous gels (Phast-system; Pharmacia).

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*Abbreviations:* D, dimensional; EDTA, ethylene diamine tetraacetate; Fo, *N*-formyl; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PAGE, polyacrylamide gel electrophoresis; PDF, peptide deformylase; SDS, sodium dodecyl sulfate.


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Detection was performed with the ECL system (Amersham) and developing was done on ECL-Hyperfilms (Amersham).

*2.4. Matrix-assisted laser desorption mass spectrometry*

Samples were prepared by mixing 1.5 μl of matrix (consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid in 40% acetonitrile–0.1% trifluoroacetic acid) with 1 μl of protein (5–10 pmol). The mixture was then loaded on a stainless steel sample holder and dried at room temperature. A VG analytical Tofspec mass spectrometer equipped with a 337 nm laser was used for sample analysis at a 25 kV acceleration voltage. 100–200 shots were accumulated from each spectrum acquisition in the positive ion mode. Calibration with external standards was obtained with a mixture of cytochrome *c* and myoglobin.

*2.5. NMR methods*

Protein samples were dried by lyophilization and resuspended in 0.4 ml (final volume) of 20 mM $KH_2PO_4$ (pH 7.25), 10% $^2H_2O$ (v/v) and 0.02% $NaN_3$ (w/v). Final protein concentrations were 4–5 mM. NMR experiments were recorded at 30°C on a Bruker AMX600 spectrometer. The 2D NOESY spectra were recorded with 512 $t_1$ experiments, each of 2048 data points over a spectral width of 7246 Hz, with the carrier on the water frequency which was suppressed by presaturation. The mixing time was of 160 ms. Using the GifA software for processing [16], data were zero-filled to 1024 points in the $t_1$ dimension. Appropriate shifted sine bell window functions were applied in both dimensions prior to Fourier transformation and the resulting spectra were subjected to a polynomial baseline correction. Spectra were analyzed with the help of the GIFC software [17].

## 3. Results and discussion

*3.1. E. coli PDF activity is left intact after C-terminal truncation*

When incubated in the presence of increasing amounts of trypsin, *E. coli* PDF, which migrates as a 23 kDa peptide, was first converted into a 20 ± 1 kDa form and subsequently into a 19 ± 1 kDa form. The latter species proved resistant to further proteolytic attack (Fig. 1A). The deformylation activity was measured in parallel (Fig. 1B). The deformylation rate, as measured after 15 min proteolysis, increased first to a maximal value of 130%. This stimulation correlated with the transient occurrence of the 20 kDa peptide. With the further accumulation of the 19 ± 1 kDa peptide, the rate of deformylation came back to the initial value of 100%. This experiment showed that the residues removed by proteolysis were dispensable for sustaining activity in vitro.

5 mg of the fully active 19 kDa proteolytic fragment of PDF was purified by anion-exchange chromatography and shown to have retained zinc (Table 1). According to mass spectrometry analysis, the fragment was homogeneous in length with a molecular mass of 16965 ± 20 Da, a value only consistent with the removal of the last 18 C-terminal residues, beyond Lys-150.

*3.2. C-terminal truncated forms of* E. coli *PDF are fully active in vivo as well as in vitro*

Series of plasmids overexpressing PDF variants with C-terminal deletions longer than that caused by trypsinolysis were constructed. First, plasmid pdef-Ec1–147 was obtained after amplification of the *fms* gene with the help of two oligonucleotides, one of which matched the C-terminal part of the enzyme while introducing an in-frame TGA stop at codon 148 followed by a *Pst*I restriction site for further cloning into pUC18. The 5′ part of the *fms* insertion exactly corresponded to that of the pUCdef vector used in previous works of the laboratory [7-9]. The vector expressed a PDF variant called Ec1–147 which lacked the residues beyond Ser-147. Plasmid pdef-Ec1–147 proved to complement at 42°C the *fms*(Ts) strain PAL421Tr, demonstrating thereby that the last 21 residues were dispensable for the in vivo function of the enzyme. The truncated enzyme could be purified to homogeneity by using the standard strategy of PDF purification. It was noticed however that this species displayed a strongly increased affinity towards the used anionic exchanger, a property which can be explained by the marked polycationic character of the removed fragment. The purified enzyme retained zinc and full deformylation activity in vitro (Table 1).

To create longer C-terminal deletions of PDF, vectors pdef-Ec1–145, pdef-Ec1–143, pdef-Ec1–141 and pdef-Ec1–139, ex-

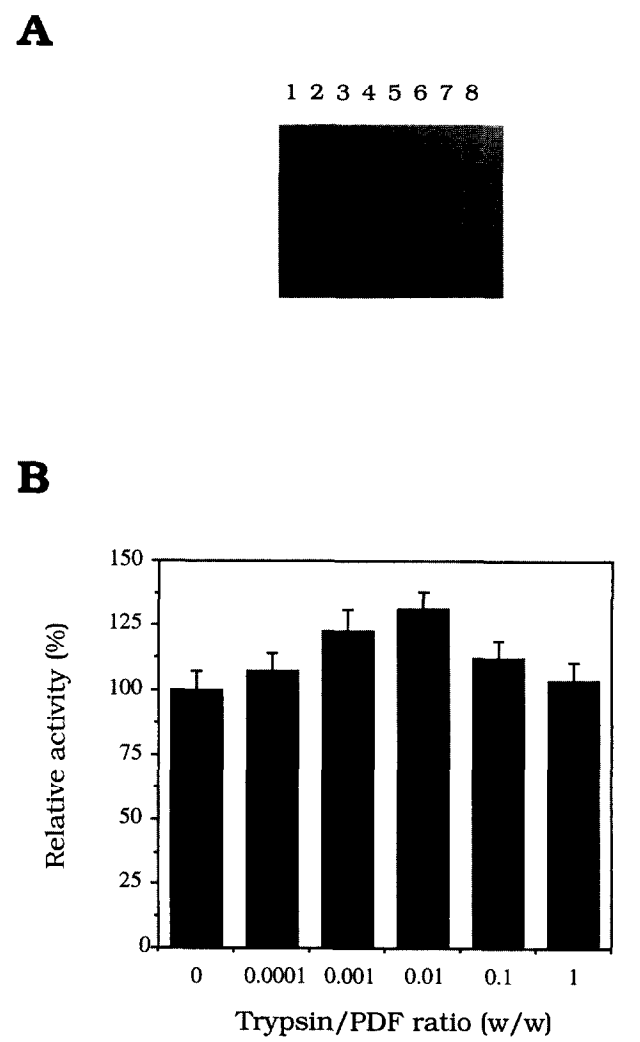


Fig. 1. Trypsin-modified PDF is fully active in vitro. 40 μg pure PDF was dialysed overnight against 0.1 M Tris (pH 7.7) plus 1 mM 2-mercaptoethanol, and then incubated in 25 μl at 37°C in the presence of increasing amounts of trypsin. The reactions were usually quenched after 15 min by the addition of chicken eggwhite ovomucoid. A: 0.5 μl of each sample was analysed by SDS-PAGE. Lane 2, no trypsin; lane 3, 4 ng; lane 4, 40 ng; lane 5, 400 ng; lane 6, 4 μg; lane 7, 8 μg and reaction carried out for 75 min instead of 15 min. Lanes 1 and 8 correspond to the low molecular weight markers of Pharmacia (94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa and 14.4 kDa). B: Each of the above samples was assayed for deformylation activity. The residual activity was measured and plotted as a function of the added amount of trypsin relative to PDF. The standard deviation on each measurement is shown by a vertical bar.

Table 1. Genetic and biochemical characterization of the effects of deletions or substitutions within the amino acid sequence of *E. coli* PDF

| Enzyme variant | Enzyme overproduction[a] | Complementation of strain PAL421Tr[b] | Zinc content[c] (mol/mol) | Relative activity (%)[d] |
|---|---|---|---|---|
| Ec | + | + | 0.8 ± 0.2 | 100 ± 10 |
| Ec S1A | + | + | 0.7 ± 0.1 | 114 ± 12 |
| Ec3–168 | + | + | 0.5 ± 0.2 | 54 ± 5 |
| Ec5–168 | ND | + | NP | NP |
| Ec7–168 | ND | − | NP | NP |
| Ec12–168 | ND | − | NP | NP |
| Ec26–168 | ND | − | NP | NP |
| Ec1–139 | ND | − | NP | NP |
| Ec1–141 | + | + | 0.5 ± 0.2 | 16 ± 2 |
| Ec1–143 | + | + | 0.6 ± 0.2 | 20 ± 2 |
| Ec1–145 | + | + | 0.7 ± 0.2 | 100 ± 10 |
| Ec1–147 | + | + | 0.8 ± 0.2 | 106 ± 10 |
| Trypsin-PDF[e] | | | 0.8 ± 0.2 | 103 ± 10 |
| Ec V138A | + | + | 0.6 ± 0.2 | 130 ± 15 |
| Ec K140A | + | + | 0.7 ± 0.2 | 110 ± 12 |
| Ec L141A | + | + | 0.6 ± 0.2 | 21 ± 2 |
| Ec F142A | + | + | 0.7 ± 0.2 | 58 ± 6 |
| Ec L141A F142S | + | + | 0.7 ± 0.2 | 11 ± 2 |
| Ec M143A | + | + | 0.6 ± 0.2 | 53 ± 5 |

[a]Production of the indicated mutant was in the range of 20 ± 5 mg homogeneous PDF obtained from 2 g harvested bacteria. When PDF could not be detected by Coomassie staining after PAGE analysis, ND (not detectable) is indicated.
[b]PAL421Tr strain was transformed at 30°C by the pUCdef plasmid expressing the indicated variant of PDF. Ampicillin resistant cells were then restreaked at 42°C on preheated LB plates containing 50 μg/ml ampicillin and 2 g/l glucose. + corresponds to plasmids allowing growth after 12 h. − corresponds to plasmids which do not allow growth after 24 h.
[c]Zinc concentrations were measured by atomic absorption spectroscopy after extensive dialysis of the samples against 20 mM Tris-HCl (pH = 7.5) and 0.1 M KCl (see section 2). The stoichiometries of zinc with respect to the protein are indicated.
[d]The deformylation activity of the zinc-free Ec L141A F142S variant is indicated in brackets. A value of 100 corresponds to 0.33 $s^{-1}$.
[e]This variant, which corresponds to the first 150 residues of PDF, was produced by mild trypsinolysis of full-length PDF (see text) and could therefore not be assayed for complementation in vivo.
NP (not purified) means that the variant could not be purified because of too low a level of production (see text).

pressing PDF with progressive C-terminal truncations of 23, 25, 27 or 29 residues, respectively, were constructed. Stop codons were introduced at the level of residues 146, 144, 142 and 140 by using a strategy similar to that described above in the case of pdef-Ec1–147. The encoded proteins were called Ec1–145, Ec1–143, Ec1–141 and Ec1–139. The Ec1–139 variant had nearly the longest C-terminal deletion compatible with the preservation of the crucial $_{132}$HEXXH$_{136}$ motif [9]. In contrast to the other three truncated variants, pdef-Ec1–139 could not relieve the thermosensitivity of the *fms*(Ts) strain (Table 1). PAGE analysis of crude extracts of JM101Tr-pdef-Ec1–139 cells did not reveal any overproduced protein by Coomassie staining. The same extract was analyzed by Western blot with antibodies raised against full-length PDF [9]. Only a very faint band, at least 20-fold less intense than that obtained with control JM101Tr-pUCdef extracts, could be detected at the expected size (not shown). These experiments suggested that the Ec1–139 species was not stable in vivo. Among the three other variants which were stable in vivo and could be purified, variant Ec1–145 showed full activity while Ec1–141 and Ec1–143 displayed specific activities significantly reduced by 6- and 5-fold respectively (Table 1).

### 3.3. *The last 21 amino acids of* E. coli *PDF are disordered in the three-dimensional structure*

The thermal stability at 50°C of fully active C-terminal truncated *E. coli* PDF (Ec1–145 and Ec1–147) was studied. Surprisingly, the thermostability of either truncated form was much greater than that of the full-length enzyme. The Ec1–147 species even showed a huge increase in stability with a $t_{1/2} \gg 700$ min, as compared to only 70 min for the full-length form. A plausible explanation was that the 23 C-terminal residues of PDF could be floppy in the structure, thereby causing this relative instability of the catalytic core. To probe this hypothesis, 2D NOESY $^1$H NMR spectra of the Ec1–147 form and of full-length PDF were compared. The spectra were fully superimposable, with virtually no additional NOESY cross-peak in the full-length PDF spectrum, as exemplified by the amide-aromatic regions of both NOESY spectra (Fig. 2). This indicated that the 3D-fold of Ec1–147 is identical to that of the full-length protein. A couple of additional amide peaks were observed in the case of full-length PDF (shown by arrows in Fig. 2), which could correspond to the side chains of either Gln-151, Gln-152 or Gln-156. Furthermore, by comparing the 1D spectra of the two forms, three additional resolved amide peaks can be recognized in the spectrum of full-length PDF (not shown). These peaks were however quite broad and do not give rise to NOESY cross-peaks in the 2D experiment. The fact that no other NOESY correlations specific of the full-length protein could be detected indicates that the protons corresponding to the C-terminal domain of full-length PDF are either in fast exchange with the solvent and/or undergo quick motions in solution. In either case, this strongly suggests that, in addition to being dispensable, the last 21 residues of PDF are disordered.

### 3.4. *Importance of the side chains of residues L141 and F142 for the activity and stability of* E. coli *PDF*

Since the removal of residues upstream of Tyr-145 caused a decrease of the activity and/or stability of PDF, it was of interest to precisely assess the contribution of each side chain in the 138–145 region of *E. coli* PDF. This could be studied by systematically substituting each side chain of this peptide by Ala. Notice that the substitution by Pro of Gly-139 and by

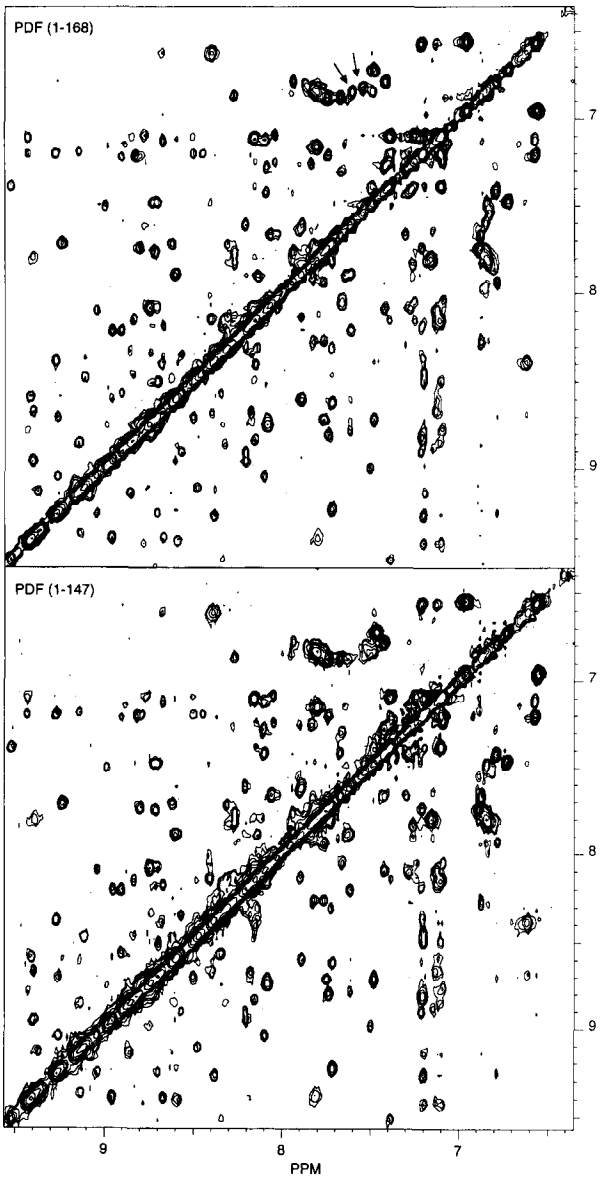


Fig. 2. $^1$H NMR 2D NOESY spectra of full-length and Ec1–147 *E. coli* PDF. Samples were buffered at pH 7.25 in 20 mM $KH_2PO_4$. Spectra were recorded at 303 K on a 600 MHz Brucker spectrometer. The amide-aromatic regions of both spectra are shown. Top: Full-length PDF; Bottom: Ec1-147 PDF. Arrows point to two additional amide cross-peaks seen in the spectrum of full-length protein.

Ala of either Asp-144 or Tyr-145 has been already reported to have no effect on the activity, the protease sensitivity and the zinc-binding properties of the enzyme [9]. In this study, the substitution by Ala of V138, K140, F142 and M143A caused no significant modification of the catalytic parameters and zinc-binding stoichiometries of PDF (Table 1). In the case of variant Ec L141A, the catalytic efficiency was reduced by 5-fold.

Since Leu-141 and Phe-142 are strictly conserved in the sequences of *T. thermophilus* and *E. coli* PDF [6], an additional mutant, Ec L141A F142S, was studied. Its catalytic efficiency of deformylation was reduced by 9-fold as compared to wild-type PDF (Table 1). However, although the zinc content of the purified enzyme was identical to that of wild-type PDF, this metal could be removed after extensive dialysis in the presence of 6 mM 1,10-phenanthroline and the zinc-free apoenzyme was inactive. It should be noted that such a removal of the zinc ion could not be observed with wild-type PDF or any studied variant of the 138–145 peptide. Since the role of the zinc ion of PDF is both catalytic and structural [8,9], the above result might indicate that residues L141 and F142 participate together in the overall structuration and activity of PDF. Consequently, the particular behavior of variants Ec L141A and Ec L141A F142S may account at least partly for the huge decrease in activity and stability in vivo accompanying the removal of the 140–145 peptide.

### 3.5. *Deletions at the N-terminal end of* E. coli *PDF inactivate the protein*

Before producing deletions of the N-terminal part of *E. coli* PDF, we first introduced several substitutions in pUCdef to create a unique *Nco*I cloning site at the level of the initiation codon. The resulting vector was called pdef-EcS1A. These changes caused the substitution of Ser-1 into Ala in the PDF sequence. The corresponding protein was purified and showed no modification of its catalytic parameters and zinc content as compared to wild-type PDF (Table 1). It could therefore be concluded that the side chain of Ser-1 was not important in the catalytic process. To create N-terminal deletions of the enzyme, the *fms* gene was amplified from pUCdef with the help of two oligonucleotides, one of which introduced a unique *Nco*I restriction site in the 5′ part of the gene at the desired codon. The amplified fragments were restricted in the presence of *Nco*I and *Pst*I and cloned within the same sites of pdef-EcS1A, yielding pdef-Ec3–168, pdef-Ec5–168, pdef-Ec7–168, pdef-Ec12–168 and pdef-Ec26–168. The five vectors were identical to pdefS1A, but missed the indicated codons at their very 5′ part. It is important to note that, in order to maintain a constant expression of the produced proteins [18] and a same extent of removal of the N-terminal methionine [19], each *fms* gene in the above constructions started with Met-Ala codons, i.e. ATGGCT. The five plasmids, encoding proteins with deletions of the N-terminal moiety of 2, 4, 6, 11 and 25 residues, respectively, were introduced in the *fms*(Ts) strain. With the three plasmids expressing the PDF with the longest deletions, the *fms*(Ts) strain PAL421Tr failed to grow at the non-permissive temperature of 42°C (Table 1). Actually PAGE analysis of crude extracts of JM101Tr cells expressing these plasmids did not reveal any overproduced protein by classical coomassie-staining. When a Western-blot analysis was carried out by using antibodies raised against the full-length enzyme [9], only very faint bands at the expected sizes, at least 100-fold less intense than that obtained with the control JM101Tr-pUCdef extract, could be detected. This meant that the deleted proteins were likely to be unstable in vivo.

With a deletion of only two residues, *E. coli* PDF was active in vivo and could be purified as a fully active species (Ec3–168, see Table 1). A further two amino acids deletion (Ec5–168) created a species which, although it was capable of complementing the *fms*(Ts) strain, was so unstable that its low overproduction yield prevented purification (Table 1).

These results clearly suggest that the N-terminal domain of PDF cannot be shortened by more than two residues without dramatic consequence on the overall stability of the protein.

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